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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Prolactin (PRL) is a hormone recognized as having both proliferative and differentiative activities in the mammary gland. Current theory proposes that it is the coexisting steroidal environment which dictates whether PRL is proliferative or differentiative. Preliminary data, presented as part of the initial proposal and established during year 1, however, suggested that it was the form of PRL released that dictated whether proliferation or differentiation would occur. By form of PRL we mean whether the PRL is released from the pituitary as an unmodified polypeptide or whether it is phosphorylated. To study this issue, we have produced recombinant, unmodified PRL (U-PRL) and a recombinant, molecular mimic of phosphorylated PRL, S179D PRL. In the studies conducted during the first and second year, we have demonstrated that U-PRL promotes mammary growth, while S179D PRL inhibits growth and promotes differentiation. Further, we have demonstrated that these effects of U-PRL and S179D PRL are produced directly on the mammary gland. In addition, we have been able to show that U-PRL and S179D PRL exert these very different effects by changing the balance of signaling between the two major pathways in mammary epithelium. Thus U-PRL primarily uses the Jak 2-Stat 5a pathway, while S179D PRL increases use of the MAP kinase pathway.

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INTRODUCTION

Prolactin (PRL) is released from the pituitary as a variety of posttranslationally modified forms (1). Work from this laboratory has concentrated on the interactive biology of the unmodified hormone and its phosphorylated counterpart, primarily because of the antagonistic activities of these two PRLs in many tissues (2-23). From the results of our studies thus far, we have developed the working hypothesis that phosphorylated PRL (P-PRL) antagonizes the growth-promoting activities of unmodified PRL (U-PRL), but that when the function of PRL in a tissue is not related to growth, the two PRLs show different degrees of the same activity. Standard PRL preparations, distributed by the NIDDK, contain a mixture of U-PRL and P-PRL and therefore produce effects which are an aggregate of the two activities. Where growth is concerned, the NIDDK standard PRL would be expected to be less efficacious than U-PRL. Where induction of a tissue-specific protein is concerned, the P-PRL would be expected to be more efficacious than the NIDDK standard PRL. In order to study the differential biology of these two PRLs, we have developed recombinant versions of each (15). The production of PRLs without posttranslational modifications was accomplished by expression in E. coli. The production of recombinant P-PRL was further accomplished by synthesizing a molecular mimic. This molecular mimic substitutes an aspartate residue for the normally phosphorylated serine (to produce S179D PRL). An aspartate residue mimics a phosphoserine both by size and charge of the side-chain. The advantage of the recombinant forms is that 1) they can be produced in sufficient quantities for *in vivo* studies; 2) they are completely free of contamination by each other; and 3) the molecular mimic of P-PRL cannot be dephosphorylated during experimental procedures. In addition, we have developed human versions of these two PRLs in order to be able to follow the effect of their administration on endogenous PRL production in experimental animals and so that we gain information about a molecule that could potentially be used to fight breast cancer in the human population. We have proposed, and our data now support, that it is a balance between the proliferative and anti-apoptotic effects of U-PRL and the anti-proliferative and differentiative effects of P-PRL that controls the end-effects of total PRL. In the mammary gland, changes in the ratios of the two PRLs would result in cyclic changes in the breast, but the proliferative actions of U-PRL would be most exaggerated during pregnancy, while the differentiative actions of P-PRL would be most exaggerated during lactation. The purpose of this project was to determine whether our prediction of the differential roles of these two PRLs in the mammary gland held true, whether these effects were independent of steroidal influences on the mammary gland and, whether treatment with the molecular mimic of P-PRL would result in refractoriness to carcinogen-induced tumors.

BODY

Statement of work item (1).

Because of the critique of the annual report in 2001, basically the same manuscript and results are described and appended for this SOW item as appeared last year. We hope that the reviewers find this manuscript, which is now in press in Cell and Tissue Research, improved.

As detailed in the manuscript in the appendix, we have determined that U-PRL and P-PRL (as mimicked by S179D PRL) have very different activities in both the pregnant and non-pregnant mammary gland. Thus, we have determined that U-PRL promotes ductal growth and branching while S179D PRL inhibits ductal branching, inhibits the growth of alveoli while at the

same time promoting β-casein gene expression. Figure 1 in the appended manuscript shows equivalent sections of mammary glands from untreated term pregnant dams (panels A & D), those treated with U-PRL (B and E) and those treated with S179D PRL (C and F). Treatment with U-PRL resulted in larger lobuloalveoli than those of the control pregnant animals, whereas treatment with S179D PRL resulted in smaller lobuloalveoli than the controls. The area occupied by alveoli versus stroma and ducts was significantly different from controls in both treatment groups (Table 1 in manuscript). Of particular note was the finding that, in the 40% smaller glands of the S179D PRL-treated animals, the area occupied by lobules was reduced to almost half ($p < 0.001$). A difference in morphological appearance of the milk/colostrum was also evident in the different treatment groups. Thus, U-PRL treatment increased the number of lipid droplets, whereas S179D PRL treatment decreased the lipid content.

Table 2 in the attached paper shows that progesterone (P), estradiol (E) and corticosterone (C) levels are unaltered by the treatments. Northern analysis of β-casein expression in these animals is shown in Figure 2. U-PRL treatment caused reduced β-casein expression, whereas S179D PRL enhanced β-casein expression. The ability of S179D PRL to enhance β-casein expression was confirmed in a rodent mammary cell line *in vitro* and this is illustrated in Figure 3.

When non-pregnant animals were exposed to the different PRLs, both resulted in mammary development by comparison to the controls. However, U-PRL did this by promoting ductal and alveolar growth, while S179D PRL by contrast, produced smaller alveoli arising from smaller ducts (Figure 4 in appended manuscript). The overall picture is best illustrated in the whole mount images shown as Figure 5. Morphometric analysis showed the ducts of the U-PRL-treated glands to be 1.4 fold the diameter of the ducts in the S179D PRL-treated and control glands (Table 3). This increase in size of the ducts was not associated with an increase in the duct's dense stroma (Table 3). P, E and C were not significantly different among the 3 groups of non-pregnant animals.

These results show a likely direct effect of the different PRLs because P, E and C were unaltered by treatment. An effect via placental products can also be eliminated since similar changes occurred in the non-pregnant animals. Thus U-PRL promotes mammary growth, while S179D PRL inhibits growth and yet promotes a differentiated function.

Statement of work item (2).

Part of our hypothesis is that exposure to P-PRL during late pregnancy and early lactation is responsible for the refractoriness to carcinogen-induced tumor formation seen in parous animals and women with an early first pregnancy. To test this hypothesis, we compared the incidence and time to tumor formation among four groups of 24 rats each. The first group had been pregnant, the second group had been pregnant with continual exposure to extra U-PRL throughout their pregnancy and for 1 week thereafter, the third group had been pregnant with continual exposure to extra P-PRL, in the form of S179D PRL for the same period of time, and the fourth group were virgin animals purchased, shipped and housed with the others. All animals had Alzet minipumps inserted subcutaneously. The dosage of the PRLs gave a circulating concentration of 50 ng/ml and had no effect on the concentration of P, E or C. Since the S179D PRL-treated animals fail to lactate and each group needed to be treated equivalently, all pups

were removed within 12h of birth. The carcinogen, N-methyl-nitroso urea (NMU) was administered (50 mg/kg IP) 7 days after delivery (i.e., when the pumps were empty). We anticipated that groups 1 and 4 would reproduce previously published findings concerning the effect of a pregnancy on refractoriness to the induction of tumors.

4th Month	5th Month	6th Month	7th Month	8th Month	9th Month	10th Month
1-12	1-12	1-12	1-12	1-12	1-12	1-12 (5X8 mm)
			1-16	1-16	1-16	1-16 (barely palpable)
1-19	1-19*	1-19	1-19	1-19	1-19	1-19 (Killed at month 5)
			1-26**	1-26	1-26	1-26 (Killed at month 7)
4-16	4-16	4-16	4-16	4-16	4-16	3-23 (43.29X32.04 mm)
						3-24 (10.72/X0.45 mm)
						4-16 (27.56/X0.21 mm)
						4-20 (33.56X24.83 mm)

Group 1, Pregnant with Saline in pump
Group 2, Pregnant with U-PRL in pump (6μg/24 h)
Group 3, Pregnant with S179D PRL in pump (6 μg/24h)
Group 4, virgin group with Saline in pump

*Killed (66.54X52.35mm, 91.29 g), **Killed (32.4X30, 29.64 g)

Table of tumor incidence and size in the treatment groups. The animal numbers are given e.g. 1-12 is an animal in group 1 which is number 12. For each month, the animals are shown in a cumulative fashion, i.e. even though animal 1-19 had to be killed at month 5 because of the size of the tumor, it stays on the chart.

Unfortunately, in the months of analyses, we found a greater number of tumors in the untreated pregnant versus the virgin group and a very low incidence of tumors overall. In retrospect, we believe that these aberrant results may have been due to two causes; increased levels of stress in the animals and a poor quality NMU. As a group, the animals arrived at the animal facility in poor condition and we had some difficulty getting them all pregnant. We did not consider the possibility that stressful shipment could have a permanent effect on our long-term experiment, but clearly it did. Because our control groups did not produce the expected result, we cannot interpret the results in the groups treated with U-PRL and S179D PRL. This experiment will be repeated taking great care to minimize stress and utilizing a different source of NMU.

Statement of work item (3).

We have been successful in establishing a rat mammary gland organ culture system. Other investigators have used mouse mammary glands for this purpose and the mouse system is well established (24). No one, however, has reported success with a rat system. We have persevered and used the rat system because it is a better animal model for the study of human breast cancer and a better animal model for endocrine-related cancer. The challenges for the rat organ system include the larger size of the gland and the difficulty in ensuring access of administered polypeptide hormone to the ductal tree as it sits on a larger fat pad. Having conducted age and dose-response studies in this system, we have concluded that the use of

glands from 21-day-old virgin females and an incubation in 15 μg/ml of each PRL will give us dependable results. We use the contralateral gland as the control for each animal. The number 4 glands have been used for whole mount analysis, and the number 5 glands for regular histology.

Using this system we have investigated direct effects of U-PRL and S179D PRL on the glands *in vitro*. Glands are incubated for 5 days in insulin (I, 5 μg/ml), aldosterone (A, 1 μg/ml), progesterone (P, 1 μg/ml) and estrogen (E, 1 ng/ml) with and without added U-PRL or S179D PRL. In the absence of either PRL, ductal growth and branching occurs (Figure 1 appended separately). In the presence of U-PRL, branching appears normal and alveoli develop (Figure 2). In the presence of S179D PRL, branching is inhibited and the ducts are smaller (Figure 3). Because the major role of P in development of the mammary gland is the stimulation of branching within the ductal tree (25), it appears that S179D PRL, not only inhibits the effects of U-PRL, but also the effects of P. Whether this is an effect via alterations in the production of mammary PRL, the number or forms of the PRL receptor, or via effects on the expression of ductal proteases or growth factors in the stroma remains to be determined. Ongoing analyses include development of a morphometric method for quantifying the observed changes in ductal branching.

Derivative experiments and results

Presentation of the data produced under the auspices of this grant has made us aware that many investigators in the field have difficulty with the concept that two different PRLs can use the same PRL receptor(s) and yet have different effects on a cell. In other words, that U-PRL can promote cell growth, while S179D PRL not only inhibits the action of U-PRL on growth, but is also capable of inducing β-casein gene expression. To overcome this problem, we have investigated the signaling pathways activated after treatment with U-PRL or S179D PRL. This part of the project, being a derivative part of the study, was partially funded from other sources. These experiments were conducted using a normal rodent mammary cell line and have recently been submitted for publication. HC11 cells are unusual among mammary cells *in vitro* because they retain their ability to respond to PRL by inducing β-casein gene expression . They therefore represent a model system in which we can investigate the differential signaling of U-PRL and P-PRL. The current consensus is that the main signaling pathways for PRL are the Jak 2-Stat 5 pathway and the MAP kinase pathway (reviewed in 26). There are, however, many more signaling molecules that are activated to some degree in a variety of tissues (reviewed in 26). From our experimentation, we were able to demonstrate that U-PRL primarily activated the Jak 2-Stat 5 pathway and that S179D PRL primarily activated the MAP kinase pathway, although both PRLs used both pathways to some extent. Figure 4 illustrates very similar Jak 2 activation with U-PRL or S179D PRL while Figure 5 illustrates very different Stat 5a tyrosine phosphorylation as a result. Even with reduced tyrosine phosphorylation in response to S179D PRL, however, electromobility shift assays showed that very similar complexes were produced between nuclear proteins and an oligonucleotide equivalent to the Stat 5 binding site of β-casein and that most of the complexes contained Stat 5a and not Stat 5b (Figure 6). Phosphoaminoacid analysis of Stat 5 showed a higher phosphoserine content in response to S179D PRL (Figure 7). Serine phosphorylation of other Stats has been shown to increase transcriptional activity (27) and hence this may also be the case for Stat 5a and the β-casein gene. Analysis of MAP kinase signaling demonstrated that S179D PRL was the better activator of ERKs 1 and 2 and that a 7-day incubation in S179D PRL upregulated ERK signaling almost 3 fold (Figure 8), while a 7-day

incubation in U-PRL upregulated Stat 5a activation 2 fold. When an inhibitor of MAP kinase, PD 98059, was included in the incubations, it inhibited the amount of β-casein expression seen in the S179D PRL-treated cells that was over and above that seen with U-PRL, but had no effect on U-PRL-stimulated β-casein gene expression or the equivalent portion of the S179D PRL response (Figure 9). Incubation in S179D PRL for 7 days also markedly upregulated expression of the short PRL receptor (Figure 10). We propose that U-PRL interacts with the long and short form of the PRL receptor such that signaling occurs primarily through Jak 2 and Stat 5 with a small amount of MAP kinase activation produced at both the long and short receptor. The different conformation of the receptors brought about by the binding of S179D PRL, however, results in reduced Stat 5a signaling and increased MAP kinase signaling. As a consequence of upregulation of the short receptor in response to S179D PRL, the MAP kinase signaling is increased. Signaling through MAP kinase results in superior β-casein gene expression. Thus, we have demonstrated that U-PRL and S179D PRL primarily use different signaling pathways (although both are required) and that long-term incubation in one PRL or the other can exaggerate the difference.

KEY RESEARCH ACCOMPLISHMENTS NOT PREVIOUSLY REPORTED

- Established a tissue explant model for the rat mammary gland
- Using the explant model,
  Produced further evidence that the different forms of PRL exert their different effects directly on the mammary gland
- Using the explant model,
  Established that S179D PRL inhibits progesterone-mediated branching in the mammary ductal tree
- Established that U-PRL and S179D PRL initiate their different effects on the mammary gland by differential use of the two major signaling pathways.

REPORTABLE OUTCOMES

- Symposium presentation at the 2001 Endocrine Society meeting, Denver, CO, entitled "Signaling and Biological Activity of a Molecular Mimic of Phosphorylated Prolactin"
- Cell and Tissue Res paper appended.
- Oral presentation by Wei Wu at the 2002 Endocrine Society meeting, San Francisco, CA, entitled "Differential Signaling of Unmodified PRL and S179D PRL in HC11 Cells"
- Development of *in vitro* rat mammary system
- A grant application to the Army Breast Cancer Program by Wei Wu to further investigate signaling and regulation of the cell cycle by U-PRL and S179D PRL, entitled "S179D Prolactin Inhibits Breast Cancer by Initiating Alternative Signaling Leading to Cell Cycle Arrest"

CONCLUSIONS

Results obtained in the past year substantiate our previous conclusions about the differential roles of U-PRL and S179D PRL in the mammary gland. In other words, that U-PRL promotes growth, while S179D PRL inhibits growth and promotes differentiated function.

Results also substantiate our previous conclusions that these differential effects are brought about by actions directly on the mammary gland. Although we previously believed that S179D PRL only inhibited the effect of U-PRL on ductal growth, further work has illustrated that S179D PRL can inhibit the effect of progesterone on mammary ductal branching. Branch points in ducts are areas of cell proliferation and matrix destruction. Both cell proliferation and matrix destruction are important for tumor growth and the metastatic potential of tumors. Analysis of the differential signaling initiated by the two forms of PRL showed that the form of PRL, which stimulates growth, mostly uses the Jak 2-Stat 5 pathway while, contrary to expectations, the form of PRL that stimulates differentiation mostly uses the MAP kinase pathway. Signaling pathways are also potential points of intervention in the fight against tumor formation and spread. An agent which inhibits proliferation, potentially inhibits matrix destruction and promotes differentiation in the mammary gland, has the potential to be an important therapeutic for the prevention and/or treatment of breast cancer. In the final year of the grant, we will repeat item "2" in the statement of work, complete item "3" and conduct items "4" and "5". Further funding will be applied for to investigate the molecular mechanisms whereby S179D PRL inhibits progesterone-induced branching.

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FIGURE LEGENDS

Figure 1: Control mammary explant cultured for 7 days in I, A, P and E. Note branching and therefore the network appearance of the ductal tree.

Figure 2: Mammary explant additionally exposed to U-PRL. Note the development of alveoli which is superimposed on a normal network of ducts.

Figure 3: Mammary gland explant additionally exposed to S179D PRL. Note the reduced branching and smaller ducts.

Figure 4: JAK 2 activation by U-PRL, S179D PRL and standard pituitary PRL. Immunoprecipitation was with anti-Jak 2. The upper panel was blotted with anti-phosphotyrosine and then stripped and reprobed with anti-Jak 2 to produce the lower panel. 0, no added PRL; N, addition of NIDDK standard human pituitary PRL, U, unmodified recombinant human PRL; S179D, recombinant S179D human PRL. Note that each PRL activates Jak 2 and that the degree of activation is very similar. This blot is representative of 3 separate experiments.

Figure 5: Stat 5a activation in response to U-PRL, S179D PRL and standard pituitary PRL. Immunoprecipitation was with anti-Stat 5a. The upper panel was blotted with anti-phosphotyrosine and then stripped and re-probed to produce the lower panel blotted with anti-Stat 5a. 0, no added PRL; N, addition of NIDDK standard human pituitary prolactin; U, addition of unmodified recombinant human PRL; S179D, addition of recombinant human S179D PRL. Note the superior tyrosine phosphorylation by NIDDK PRL and U-PRL and the much weaker activation by S179D PRL. This blot is representative of 8 separate experiments.

Figure 6: EMSA analysis of the β-casein GAS site following stimulation with U-PRL, S179D PRL and standard pituitary PRL. After stimulation of the cells with each PRL, nuclear extract proteins (NE) were incubated with radiolabeled GAS site oligonucleotide with or without competition by anti-Stat 5a (α 5a), anti-Stat 5b (α 5b) or 100 fold unlabeled oligonucleotide (+ com). 0, no added PRL; OV, addition of ovine PRL; N, addition of NIDDK standard human pituitary PRL; U, addition of unmodified recombinant human PRL; S179D, addition of recombinant human S179D PRL. Note the very similar complexes produced by NIDDK PRL, U-PRL and S179D PRL and that formation of complexes in the latter two (only two tested) could

be competed for by anti-Stat 5a, but not anti-Stat 5b. This autoradiogram is representative of 4 separate experiments.

Figure 7: Tyrosine and serine phosphorylation of STAT 5 in response to U-PRL, S179D PRL and standard pituitary PRL. The bars show the means ± SE of 3 separate phosphoamino acid analyses from one radiolabeling experiment and are expressed as relative densitometric units. Tyr, phosphotyrosine; Ser, phosphoserine; 0, no added PRL; N, addition of NIDDK standard human PRL; U, addition of recombinant human unmodified PRL; S179D, addition of recombinant human S179D PRL.

Figure 8: Activation of ERK 1, ERK 2 and Stat 5a in response to U-PRL and S179D PRL, both before (Panel A) and after (Panel B) a 7-day incubation in those PRLs. The cells were incubated for 15 min for panel A. For panel B, the cells were incubated in the PRLs for 7 days, the PRLs were withdrawn for 2h and then reapplied for 15 min. The upper panels (a) show the result of immunoprecipitation with anti-total ERK and blotting with anti-active ERK. Below them is the same blot stripped and reprobed with anti-total ERK (b). The third panel down shows the result of immunoprecipitation with anti-Stat 5a and blotting with anti-phosphotyrosine (c) and the lowest panel, the result of stripping and reprobing with anti-Stat 5a (d). In each case, the A panels and B panels were exposed to the same film so that direct comparisons could be made from before and after the 7 day incubation. Note the greater activation of ERKs 1 and 2 by S179D PRL and the greater activation of Stat 5a by U-PRL. Also note the upregulation of ERK signaling after the 7-day incubation in S179D PRL and downregulation of Stat 5a signaling. These blots are representative of 5 separate experiments.

Figure 9: Expression of β-casein mRNA in response to U-PRL and S179D PRL in the presence and absence of the MAP kinase inhibitor, PD98059. HC11 cells were incubated in the PRLs for 7 days in the absence or presence of PD 98059 (PD). 0, no addition of PRL; 0 + PD, PD alone; U, addition of unmodified recombinant human PRL; U + PD, addition of unmodifed recombinant human PRL plus PD; S179D, addition of recombinant human S179D PRL; S179D + PD, addition of recombinant S179D human PRL plus PD; The data are derived from Northern blots, normalized to 18S rRNA and are expressed as the mean ± SE of three separate experiments. * = $p < 0.01$, # = $p < 0.05$ *versus* S179D PRL. Note that PD had no effect on U-PRL-stimulated expression while it inhibited the additional stimulation brought about by S179D PRL.

Figure 10: Expression of the short PRL receptor in response to a 7 day incubation in U-PRL or S179D PRL. 0, no added PRL; U, addition of recombinant unmodified human PRL; S179D, addition of recombinant human S179D PRL. The data are derived from Northern blots, normalized to 18S rRNA and are presented as the mean ± SE from 5 separate experiments. * = $p < 0.01$, # = $p < 0.05$ *versus* S179D PRL. Note the doubling in the expression of the short receptor in response to S179D PRL.

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Regular Article

Pseudophosphorylated prolactin (S179D PRL) inhibits growth and promotes β-casein gene expression in the rat mammary gland

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Abstract. We have investigated the individual roles of unmodified prolactin (U-PRL) and a mimic of phosphorylated PRL (S179D PRL) in mammary development. Recombinant versions of the PRLs were delivered to rats throughout pregnancy at a rate of 6 μg/24 h per rat and to non-pregnant females at a rate of 24 μg/24 h per rat. Measurement of progesterone, corticosterone, and estradiol showed no effect of the administered PRLs on the levels of these other mammotropic hormones. Histological and morphometric analysis showed U-PRL to cause mammary growth, whereas S179D PRL inhibited growth . Molecular analysis demonstrated decreased β-casein expression in the mammary glands of the U-PRL-treated animals at term and increased β-casein expression in the mammary glands of the S179D PRL-treated animals. Superior β-casein gene expression in response to S179D PRL versus U-PRL was confirmed in HC11 cells. We conclude that U-PRL is important for growth, whereas S179D PRL promotes at least one measure of differentiated function in the mammary gland.

Keywords. Unmodified prolactin - Phosphorylated prolactin - Duct - Alveolus - β casein - Rat (Sprague-Dawley)

Introduction

Prolactin (PRL) has long been described as a hormone important for both growth and differentiation in the mammary gland, but its precise contribution to each of these processes has been difficult to ascertain. This is attributable, in part, to complications introduced into experimental protocols by the luteotropic action of PRL in rodent models (Hsueh et al. *1984*) and, in part, in our opinion, because PRL has been thought of as a single substance. In this study, we have used an experimental approach that maintains the normal progesterone levels of pregnancy to test the effects of increased unmodified PRL and phosphorylated PRL on mammary gland development. PRL is produced in a variety of post-translationally modified forms. We have focused our attention on the individual biological roles of unmodified PRL and phosphorylated PRL, because these two forms between them constitute 98%-100% of secreted pituitary PRL in the rodent (Oetting and Walker *1986*; Ho et al. *1993a*, *1993b*), which serves as our experimental model. They also have been demonstrated to have distinct biological activities (Ho et al. *1989*; Krown et al. *1992*; Wang and Walker *1993*; Coss et al. *1999*, *2000*; Yang et al. *2001*), the proportion of each released from the pituitary is physiologically regulated (Ho et al. *1993a*, *1993b*), and phosphorylated PRL has been found in all species thus far examined (reviewed by Lorenson and Walker *2001*). Standard preparations of PRL, such as those distributed by the NIDDK, contain a mixture of unmodified and phosphorylated PRL (Krown et al. *1992*; Wang and Walker *1993*). Any biological activity observed as a result of treatment with these preparations therefore represents an aggregate activity related to the relative proportions of the unmodified and phosphorylated PRL present (Krown et al. *1992*; Wang and Walker *1993*).

In order to determine the individual activities of unmodified and phosphorylated PRL in the mammary gland, we have administered recombinant versions of each to pregnant and non-pregnant animals, thereby altering the normal ratio of the different PRL forms in the animal. In the case of phosphorylated PRL, we have produced a molecular mimic by substituting an aspartate residue for the normally phosphorylated serine, thereby producing S179D PRL (Wang et al. *1996*; Chen et al. *1998*). Aspartate mimicry of serine phosphorylation is used extensively in studies of enzymes activated or deactivated by phosphorylation, and in several instances, extensive structural analyses have confirmed complete three-dimensional and functional mimicry (Thorsness and Koshland *1987*; Wittekind et al. *1989*). The recombinant wild-type hormone (U-PRL) is identical to unmodified PRL with the exception, like S179D PRL, of an N-terminal extra methionine (Chen et al. *1998*). The S179D PRL effectively mimics the naturally phosphorylated molecule by acting, like phosphorylated PRL, as an extremely effective antagonist to U-PRL-induced Nb2 cell proliferation (Wang and Walker *1993*; Chen et al. *1998*).

Whereas S179D PRL is an antagonist to growth at physiological ratios with U-PRL and at concentrations physiologically relevant for a given tissue (Chen et al. *1998*; Yang et al. *2001*), it is a partial agonist when used alone at concentrations 100-fold those of U-PRL (Bernichtein et al. *2001*). This finding has lead to some controversy about the molecule. There are however a number of examples of partial agonists that act physiologically as antagonists in some tissues and as very effective agonists in others. Tamoxifen and Raloxifen, for example, act as antagonists in the breast and as agonists in the bone (MacGregor and Jordan *1998*). In a similar manner, S179D PRL acts as an antagonist in the lung and thymus (Chen et al. *1998*; Yang et al. *2001*), but like U-PRL in the bone (Coss et al. *2000*). Indeed, it seems unlikely that the body would produce an absolute antagonist. Thus, it is not entirely unexpected that the biology of S179D PRL, as a mimic of phosphorylated PRL, is

more complicated than that of a simple antagonism of the actions of U-PRL. The most important reason for using the molecular mimic rather than the naturally phosphorylated molecule is in order to prevent the possible interconversion of phosphorylated PRL to U-PRL by body phosphatases. Even though this is a very slow process (Krown et al. *1992*; Wang and Walker *1993*), conversion would severely complicate interpretation of results. The aim of the present study has been to determine the individual roles of the two forms of PRL in the mammary gland by analyzing effects on structural development and expression of the major milk protein, β-casein.

Materials and methods

Animal experiments

Virgin female Sprague-Dawley rats (n=35, 16 weeks old) were divided into four groups. Five rats served as non-pregnant controls, 10 rats as normal pregnant controls, 10 rats as recipients of U-PRL, and 10 rats as recipients of S179D PRL. Alzet minipumps (Alza, Palo Alto, Calif.) delivering 6 µg PRL/24 h per rat for 28 days were implanted subcutaneously the morning after vaginal plug observation. This was considered as being day 0.5 of pregnancy. On day 6.5, blood was obtained from the tails and collected into heparinized tubes. At term, dams were separated from their pups for 20 h prior to sacrifice to standardize the histological appearance of the glands in each group. After sacrifice, the inguinal mammary glands were dissected out, measured, and processed for whole-mount or histological examination. Size was calculated by multiplying the length by the average width by the average depth. In an essentially duplicate experiment, blood samples (~ 1 ml) were taken from the tails on days 6.5 and 11.5 and trunk blood from the neck after decapitation at day 19.5. No animal was bled more than once from the tail in order to keep stresses during pregnancy to a minimum. At the time of death (day 19.5 of pregnancy, five dams in each group; or day 21.5 shortly after pup delivery, five dams in each group), inguinal mammary glands were snap-frozen in liquid N_2 for later RNA extraction. In a third and fourth experiment, non-pregnant females were treated with 24 µg PRLs/24 h per rat for 4 days. By using a higher dose for a shorter interval, it was thought likely that individual responses to each form of PRL would be exaggerated and therefore become clearer. At the time of sacrifice, trunk blood was collected, and the inguinal mammary glands were again processed either for whole-mount or regular histology. All animal procedures were approved by the University of California, Riverside Campus Committee on Laboratory Animal Care and were in accord with NIH guidelines.

Recombinant PRLs

Both recombinant human U-PRL and S179D PRL were produced and characterized as previously described (Chen et al. *1998*). Both proteins were expressed and purified in parallel and were expressed at similar levels (Chen et al. *1998*). The preparations were then tested for their activity in an Nb2 bioassay. U-PRL promoted Nb2 cell proliferation, whereas S179D PRL (like naturally phosphorylated PRL; Wang and Walker *1993*) antagonized it (Chen et al. *1998*). The PRL preparations were concentrated to 1 mg/ml saline by using Amicon Centripreps (Amicon, Danvers, Mass.) and loaded into model 2004 (first two experiments) or 2001 (third and fourth experiments) Alzet minipumps.

Histological analysis

Mammary glands were fixed in periodate-lysine-paraformaldehyde fixative (McLean and Nakane *1974*) at 4°C overnight. The fixed tissue was dehydrated in a graded ethanol series, cleared in Hemo De, and then embedded in Paraplast. Sections (6 μm thick) were cut and stained with hematoxylin and eosin. For morphometric analysis of the glands from non-pregnant animals, entire mammary glands were serially sectioned. Stained sections were viewed at a constant magnification by using a PAXIT digital image system (Midwest Information Systems, Franklin Park, Ill.), and the glands were measured on a screen. Every duct and associated dense stroma was measured on each section. The area occupied by alveoli was also measured. For morphometric analysis of term pregnant glands, three mid-gland sections from five animals in each group were analyzed. In each case, a random view at the same low magnification was photographed, and the area on each photograph occupied by alveoli versus stroma plus ducts, was determined. For whole-mounts, glands were spread on glass and then fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 60 min at room temperature. They were then washed in ethanol and defatted overnight in acetone. Defatted glands were then rehydrated and stained with carmine alum overnight at 4°C, dehydrated, and cleared in methyl salicylate before mounting.

Hormone assays

All steroid hormones were measured by radioimmune assay with a kit from Diagnostic Products (Diagnostic Products Coat-a-Count, Los Angeles, Calif.). Progesterone levels were measured in serum (trunk blood) or plasma (tail blood). Previous work had demonstrated equivalent recognition of progesterone in rat serum and plasma with heparin as the anticoagulant (Coss et al. *2000*). All results presented in a single table were produced in the same assay. Errors were therefore limited to intra-assay variation. The coefficient of intra-assay variation for this assay was 6.7% in our hands. Only trunk blood samples were assayed for corticosterone. As for progesterone, all results in the relevant table were produced in the same assay. The coefficient of intra-assay variation was 6.3% in our hands. Total estradiol levels were measured in trunk blood. The coefficient of intra-assay variation was 5.7% in our hands.

Northern blot analysis for β -casein gene expression

Total RNA was isolated from mammary tissue or HC11 cells by using the Trizol RNA reagent (Gibco BRL, N.Y.). The isolated RNA was treated with DNase I (Gibco BRL, Gaithersburg, Md.). Equal amounts of RNA (10 μg) from control and test samples were loaded on a 1.0% agarose formaldehyde gel. The gels were run at 60 V for 3-5 h. RNA was blotted onto nylon filters (Micron Separations, Westboro, Mass.) by capillary transfer with 10 × SSC (1 × SSC=150 mM NaCl, 15 mM sodium citrate, pH 7.0) and fixed by ultraviolet cross-linking. The 201-bp probe used for hybridization was from a mouse β -casein cDNA polymerase chain reaction product. The primers were: 5'-CCC GTC CCA CAA AAC ATC C-3' (forward); 5'-ATT AGC AAG ACT GGC AAG GCT G-3' (reverse). The probe was labeled with 50 μCi [α -^{32}P] dCTP (ICN Biomedicals, Costa Mesa, Calif.) by using a DECA Prime II DNA Labeling Kit (Ambion, Austin, Tex.). The labeled probes were separated by ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech, Piscataway, N.J.). After a 2-h prehybridization at 65°C with the hybridization solution (25 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, 7% SDS), hybridizations were carried out at 65°C for 16-24 h. The filters were then washed in alternating solutions of 20 mM Na_2HPO_4, pH 7.2, 1 mM EDTA, 5% SDS and then 20 mM

Na_2HPO_4, pH 7.2, 1 mM EDTA, 1% SDS for a total of three times in each. Filters were exposed to Fuji medical X-ray film (Fuji Medical Systems, Stamford, Conn.) for 1-7 days at -70°C. Probe-stripping was performed by heating the nylon filter at 95°C for 10-30 min in a solution of 10 mM TRIS-HCl, pH 8.0, 1 mM EDTA, 1% SDS. A mouse 18S rRNA cDNA fragment (DECA template-18S-mouse, 1212 bp; Ambion) was used to normalize for errors in RNA loading and transfer. A Kodak 1D Image Analysis System was used for quantification (Eastman Kodak, Rochester, N.Y.).

Effect of U-PRL and S179D PRL on β-casein gene expression in HC11 cells

RPMI 1640 basal medium (Gibco BRL, Grand Island, N.Y.) containing 2 mM L-glutamine and 2 g/l $NaHCO_3$ served as a basal medium. HC11 cells, a cloned mouse mammary epithelial cell line (Ball et al. *1988*), were grown in RPMI 1640 growth medium containing 10% fetal calf serum (Gibco BRL, Grand Island, N.Y.), 5 µg/ml insulin (Sigma, St. Louis, Mo.), 10 ng/ml epidermal growth factor (Gibco BRL, Gaithersburg, Md.), 100 U/ml penicillin, and 100 µg/ml streptomycin. Once HC11 cells became confluent, they were grown for three more days in growth medium. The medium was changed daily. On the third day post-confluency, the growth medium was removed, and the cells washed five times with RPMI 1640 basal medium. The cells were refed with priming medium. Priming medium was RPMI 1640 basal medium supplemented with 10% charcoal-stripped horse serum (Cocalico Biologicals, Reamstown, Pa.), 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml insulin, and 1 µg/ml hydrocortisone (Sigma). The cells were kept in priming medium for 24 h. The cells were then refed with induction medium. The induction medium was priming medium to which 1 µg/ml of the appropriate PRL was added. This protocol essentially follows Taverna et al. (*1991*). In the present study, cells were maintained in induction medium for 7 days and refed daily. The cells were collected for RNA isolation.

Statistical analysis

Analysis of variance was performed using the INSTAT program (GraphPAD Software, San Diego, Calif.). Post-tests comparing each potential pair of groups were performed. Bonferroni corrections were used to allow for more than one comparison against a single control group. A *P*-value less than 0.05 after Bonferroni correction was considered significant.

Results

Pregnant animals

Gross observations

Treatment with U-PRL at 6 µg/24 h per rat throughout pregnancy resulted in glands that were 1.5-fold the size of those from the untreated pregnant animals (102±7 mm^3, U-PRL; 67±5 mm^3, control; $P<0.01$). Treatment with S179D PRL at 6 µg/24 h per rat throughout pregnancy, by contrast, resulted in glands that were 40% smaller (40.2±4.6 mm^3; $P<0.05$). As reported previously (Yang et al. *2001*), the average number of pup implantation sites per animal (13±1) was indistinguishable among groups.

Histological observations

Figure 1 shows equivalent sections of mammary glands from each group of animals from the first experiments. Treatment with U-PRL resulted in larger lobuloalveoli than those in the control pregnant animals, whereas treatment with S179D PRL resulted in smaller lobuloalveoli than in the controls. The area occupied by alveoli versus stroma and ducts was significantly different from controls in both treatment groups (Table 1). Of particular note was the finding that, in the 40% smaller glands of the S179D PRL-treated animals, the area occupied by lobules was reduced to almost half ($P<0.001$). In other words, a greater amount of intervening connective tissue was evident. This had resulted in lactational failure in previous experiments where this was monitored. A difference in morphological appearance of the milk/colostrum was also evident in the different groups. Thus, U-PRL treatment increased the number of lipid droplets, whereas S179D PRL treatment decreased the lipid content (compare D-F in Fig. 1).

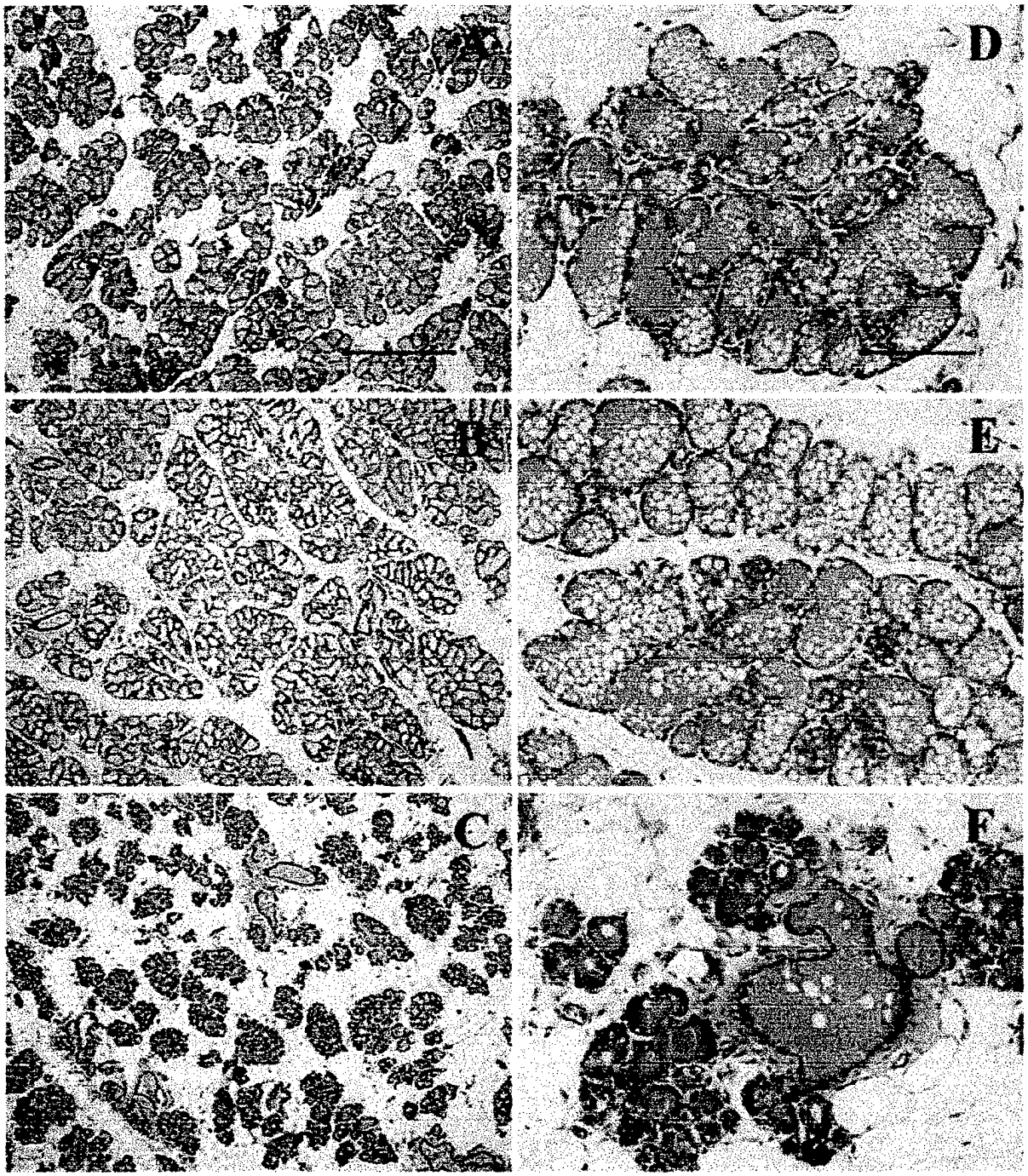

Fig. 1A-F. Term mammary glands from each treatment group. **A, D** Control rats. **B, E** Rats treated with U-PRL. **C, F** Rats treated with S179D PRL. Note the large number of lipid droplets in **E** and their relatively low abundance in **F**. Also note the proximity of the small alveoli to a relatively large duct in **F**. **A-C** × 40, **D-F** × 200. *Bar* 1 mm **(A)**, 200 μm **(D)**

Table 1. Area of gland section occupied by alveoli or stroma plus ducts on day 21.5 and expressed as a percentage of the total area. Data are expressed as the mean±SE with n=15 per group (significant difference: *P<0.05, **P<0.001)

Tissue	Day	Control	U-PRL	S179D PRL
Alveoli	21.5	67.5±1.1*,**	72.3±1.3*	38.3±0.9**
Stroma plus ducts	21.5	32.5±0.6	28±0.9	61.7±1.1

Hormone levels

Table 2 shows progesterone levels to be unaltered by U-PRL or S179D PRL treatment throughout pregnancy, and estradiol and corticosterone levels to be unaltered on day 19.5 of pregnancy. *P*-values were always greater than 0.05.

Table 2. Levels of mammotropic steroids during pregnancy in the three treatment groups of the second experiment. Data are expressed as the mean±SE with n=5 animals per time point and group. There were no statistically significant changes

Treatment	Day	Control	U-PRL	S179D PRL
Progesterone (ng/ml)	6.5	374±37	351±20	323±28
	11.5	369±20	380±20	389±43
	19.5	377±23	283±28	328±18
Estradiol (pg/ml)	19.5	15±1	14.3±1	15.1±2
Corticosterone (ng/ml)	19.5	138±29	205±62	169±22

β -Casein expression

The result of Northern analysis for β -casein expression in the day 19.5 and 21.5 samples is shown in Fig. 2. Both time points gave the same result, and so the data were combined allowing for sufficient samples for adequate statistical analysis. U-PRL treatment caused reduced β -casein gene expression, whereas S179D PRL treatment caused an enhancement. Because treatment with the PRLs was over and above the animal's own lactogens, and because normalization to ribosomal RNA can be questioned in glands with different epithelial to connective tissue ratios, we also examined the effects of the two PRLs on β -casein gene expression in the HC11 mammary cell line. Figure 3 clearly shows that S179D PRL is many-fold more effective at inducing β -casein gene expression than is U-PRL in the 7-day treatment period.

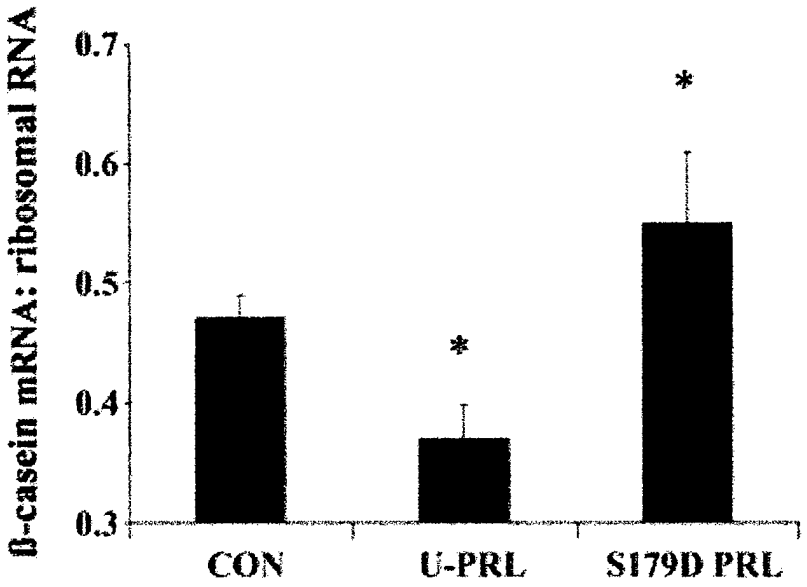

Fig. 2. Northern analysis of β -casein mRNA expression in mammary glands at days 19.5 and at term (n=8 rats per group). Amounts of β -casein mRNA are normalized to the amount of ribosomal RNA.

Results are expressed as the mean±SE (*CON* control rats receiving no additional PRL). Differences among groups were analyzed by ANOVA and individual t-tests with Bonferroni corrections. *P<0.05 (significantly different from the control group)

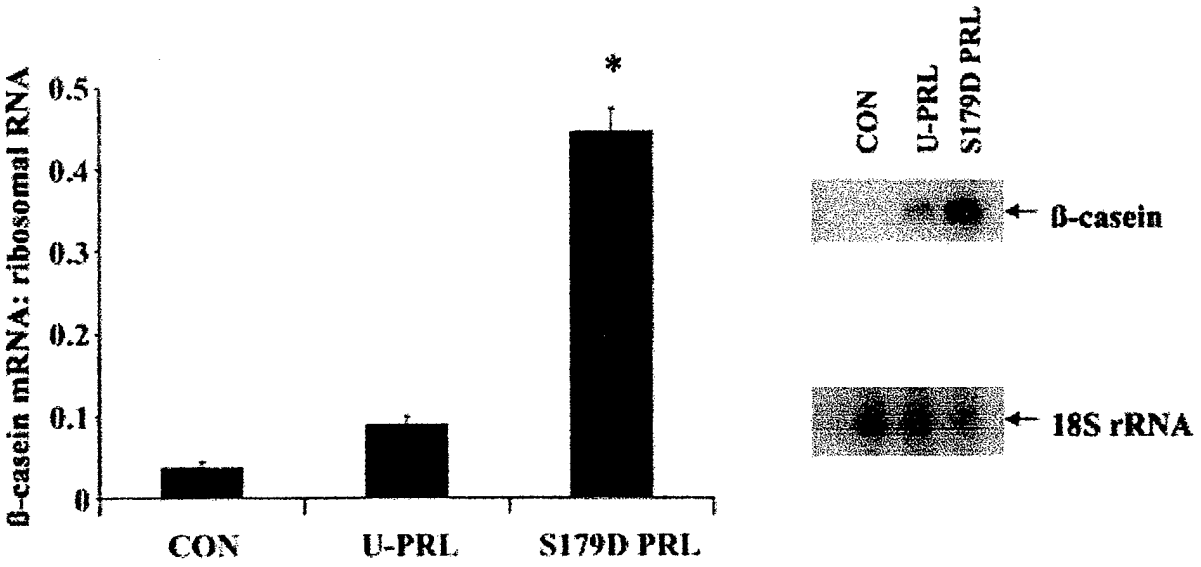

Fig. 3. Northern analysis of β -casein mRNA expression in HC11 cells in response to the various PRLs (*CON* control rats receiving no additional PRL). Amounts of β -casein mRNA are normalized to the amount of 18S RNA. Differences among groups were analyzed by ANOVA and individual *t*-tests with Bonferroni corrections. *P<0.001 (significantly different versus the control and U-PRL groups; mean±SE of five separate experiments). *Inset* Representative blot

Non-pregnant animals

By performing similar experiments in non-pregnant animals, it was possible to test whether the effects observed on the pregnant mammary gland were secondary to effects on placental lactogens. In addition, we could ask whether pregnancy levels of progesterone were required to observe these effects. The histology of mammary glands from animals treated with the PRLs at 24 µg/24 h per rat for 4 days is presented in Fig. 4. Both PRLs caused mammary development by comparison with the controls. However, U-PRL did this by promoting ductal and alveolar growth . S179D PRL, by contrast, produced smaller alveoli arising from smaller ducts. In other words, when S179D PRL was present in excess, it was a poor agonist for mammary growth but did result in some alveolar development. The overall picture is best illustrated by the whole-mount images shown in Fig. 5. Since these are of rat glands rather than of the much smaller mouse glands, whole-mount images have fewer elements in focus in a given image. Nevertheless, it is clear that the alveoli are smaller in the S179D PRL-treated glands. Because of the degree of development of these glands, it was not possible to illustrate the effects on ductal branching by using low-magnification whole-mount photographs. Instead, overall growth of the ductal tree was assessed by measuring ductal diameter. As the tree enlarges, so does the average diameter of the branches. Morphometric analysis of serial sections showed the ducts of the U-PRL treated glands to be 1.4-fold the diameter of the ducts in the S179D PRL treated and control glands (Table 3). The width of the associated dense stroma was however the same in both groups. Thus, effects on ductal growth do not seem to be secondary to proliferation of the associated dense stroma.

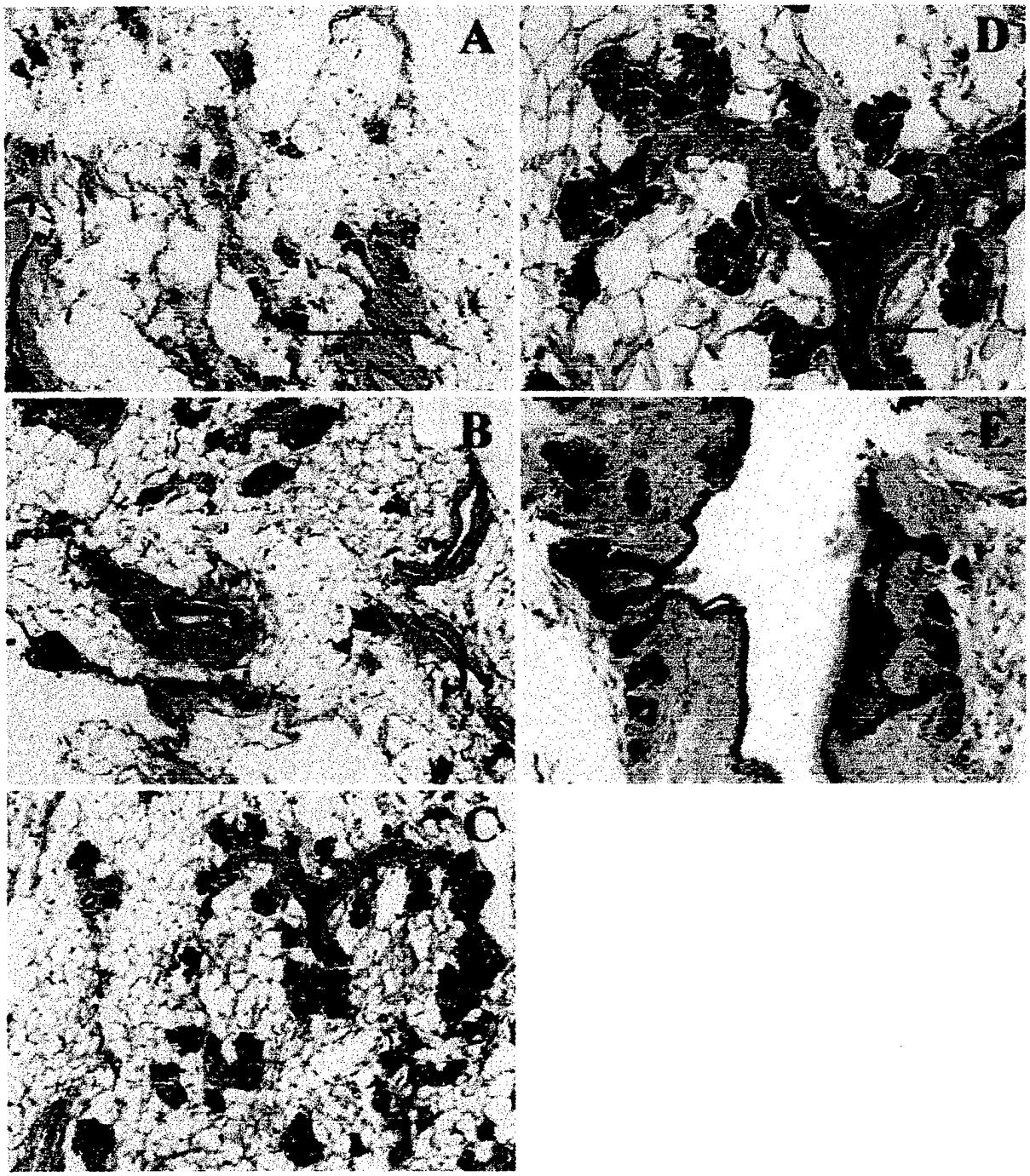

Fig. 4A-E. Non-pregnant mammary glands treated with the various PRLs for 4 days. **A** Control rats. **B** Rats treated with U-PRL. **C** Rats treated with S179D PRL. **D, E** Higher magnification views of mammary glands from rats treated with S179D PRL. Note the many small alveoli closely associated with a relatively large duct in **C-E**. **A-C** × 80, **D, E** × 200. *Bar* 500 μm (**A**), 200 μm (**D**)

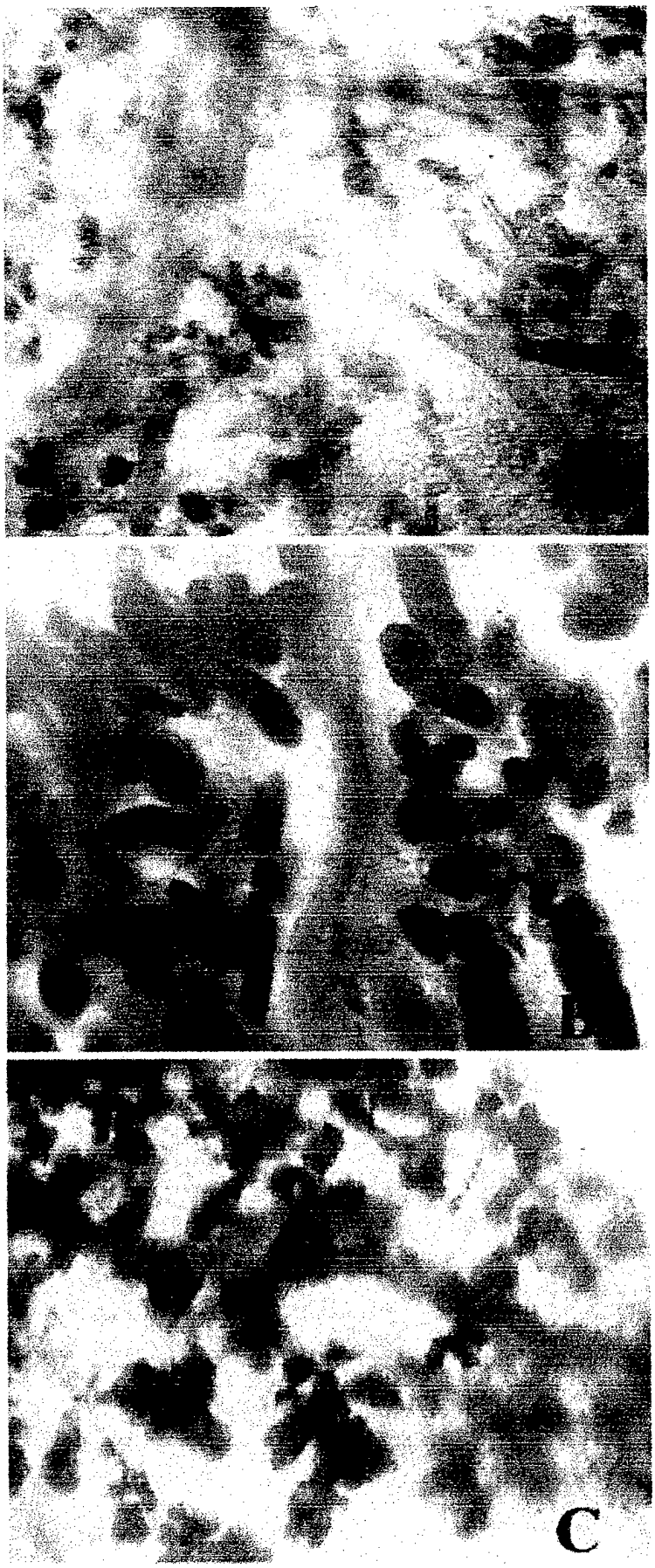
C

Fig. 5A-C. Whole-mount glands at equivalent magnifications from non-pregnant animals not treated or treated for 5 days with the various PRLs. **A** Control. **B** Treatment with U-PRL. **C** Treatment with S179D PRL. Note the larger alveoli in **B** and the multiple smaller alveoli in **C**. ×✝✝

Table 3. Duct diameters and associated dense stroma widths in cross-sections in control and the 4-day-treated non-pregnant animals. Numbers are in relative units and are expressed as the mean±SE. All cross-sectional views of 54 sections per gland for the treated groups and 30 sections per gland for the control group were analyzed. Twice as many control glands were used for the analysis so that the number of duct cross sections were similar in each group. *P<0.05, i.e., statistically significantly different from control and S179D PRL treated group

Treatment	Duct	Stroma	Ratio (stroma:duct)
Control	7.9±3.6	21.7±4.5	2.7
U-PRL	11.18±1.4*	22.12±1.5	2.0
S179D PRL	8.5±1.3	23.33±1.9	2.7

Table 4 shows no statistically significant effect of the two PRLs on corticosterone, progesterone, or estradiol levels in these non-pregnant animals. Trends were, if anything, toward equally reduced corticosterone and progesterone with each PRL, i.e., toward an equal reduction in these other mammotropic hormones with both treatments.

Table 4. Levels of progesterone, corticosterone, and estradiol in the 4- to 5-day-treated non-pregnant animals. Data are expressed as the mean±SE. These levels are not significantly different among groups (P>0.05)

Treatment	Control	U-PRL	S179D PRL
Progesterone (ng/ml)	25.2±8.0	14.6±5.3	17.4±4.4
Corticosterone (ng/ml)	331±85	280±32	256±66
Estradiol (pg/ml)	17.2±2.1	16±1.9	18.3±1.3

Discussion

Administration of additional U-PRL has markedly different effects on mammary gland histology from those seem following the administration of S179D PRL. We can conclude therefore that the effects are not attributable to a simple elevation in total PRL but are, indeed, specific to each form. Since there is no effect on progesterone, estradiol or corticosterone, we can conclude that the effects observed are not secondary to changes in these other mammotropic hormones. Similar effects in pregnant and non-pregnant animals demonstrate that these effects are not secondary to changes in placental lactogen. This has been considered as a possibility because other investigators have demonstrated that mammary gland development is related to the number of developing pups (Nagasawa and Yanai *1971*), and because the levels of placental lactogens are very much in excess of PRL in the later stages of pregnancy (Robertson and Friesen *1981*). Placental lactogens are thought to function via the PRL receptor (Freemark et al. *1993*). Similar effects in pregnant and non-pregnant animals also tell us that pregnancy levels of progesterone are not required in order to be able to see the differential activities of each form, although our current experiments do not address the question of the necessity for some

progesterone or the promotion of the processes by progesterone. Progesterone has been shown to upregulate PRL receptors on rodent mammary epithelium (Edery et al. *1985*) and hence is likely to make the system more responsive to PRL, in addition to having substantial and totally independent effects.

U-PRL significantly promotes ductal growth (as reflected in the diameter of ducts) in only 4 days at 24 μg/24 h in non-pregnant animals. This rate of administration results in circulating levels of 200 ng/ml by day 4, although days 1-3 have lower amounts as the PRL from the mini pump slowly equilibrates with tissue and blood compartments (Coss et al. *2000*). At only 6 μg/24 h, or 50 ng/ml (Coss et al. *2000*), administered U-PRL results in a 50% increase in the overall size of the mammary gland at term. Some of this size increase is attributable to the growth of lobuloalveoli, but some has to be the result of ductal growth in accord with the findings in non-pregnant animals. Since the gland as a whole is still contained within the fat pad, some general (as opposed to duct-associated dense) stromal proliferation is likely to have occurred. A similar concentration of circulating S179D PRL reduces the size of the mammary gland at term, i.e., it inhibits ductal growth and branching. In the short-term experiment, alveoli can be seen developing almost directly from large ducts in Fig. 4D, E, i.e., they appear to cap duct branch points and prevent their further development. At the same time, S179D PRL promotes β-casein gene expression. However, because the gland is too small, insufficient milk is produced to feed the pups.

When viewing the β-casein expression data from the pregnancy experiment, it is important to remember that the effect is caused by the administration of the recombinant PRLs over and above the rat's own PRL, which is a mixture of unmodified PRL and phosphorylated PRL. Changing the ratio by increasing U-PRL decreases β-casein gene expression, because it reduces the relative amount of phosphorylated PRL, which is a much better stimulator of β-casein expression. Thus, the pregnancy data are concordant with the effects of the individual PRLs on the HC11 cells.

From these results, it appears that U-PRL promotes overall growth of the mammary gland. S179D PRL, and presumably therefore phosphorylated PRL, acts as an antagonist to mammary growth but promotes an alveolar differentiated function. When used in high enough concentration, S179D PRL can also promote alveolar development. Whether this is partial agonism or promotion of differentiation remains to be established. Until now, the effects of PRL on growth versus differentiation in the mammary gland have been thought to be attributable to a change in the steroidal environment between pregnancy and lactation. This certainly plays a major role, but it is also clear that the forms of PRL are important. In regard to the forms of PRL, we have previously shown an increase in the ratio of U-PRL to phosphorylated PRL to occur during the latter two-thirds of rodent pregnancy when the mammary gland is growing (Ho et al. *1993b*). Just before parturition, there is a peak of PRL (Fliestra and Voogt *1997*), which is high in phosphorylated PRL (unpublished data); phosphorylated PRL is very high in colostrum and milk (Ellis and Picciano *1993*; Kacsoh et al. *1993*), and the majority of PRL receptors are on the milk face of the mammary epithelium (Clevenger et al. *1995*). Thus, the ontogeny of PRL forms during pregnancy and lactation is concordant with the observed effects of the individual PRL forms on the mammary gland; first, mostly growth, and then, an increase in β-casein gene expression.

Recent work utilizing a variety of mammary epithelial and stromal transplant recombinations from the PRL receptor knockout mouse and the progesterone receptor knockout mouse supports our findings of a role for a lactogen in both ductal and alveolar growth, although the ductal growth is deduced in these

studies to be indirect via effects on progesterone (Ormandy et al. *2001*). Whereas the transplant studies show that progesterone plays a very important role in ductal growth, transplant studies are qualitative and not quantitative and could easily have missed the additional, more minor, contribution of PRL itself. In this regard, a recent study by Sasaki et al. (*2001*) has also demonstrated co-operative effects of prolactin and progesterone during mouse mammary gland branching morphogenesis.

Our results showing the effects of PRL on ductal growth without effects on progesterone levels suggest a direct effect of PRL on the duct. Indeed, if there is any trend in the U-PRL-treated animals showing greater ductal growth, it is for a decrease in pregestone levels. A direct effect of PRL on ductal growth is very much in keeping with the presence of PRL receptors in ductal epithelium (Ouhtit et al. *1993a*). Alternatively or additionally, PRL may act indirectly via the stroma, although it is clear that the amount of dense stroma is not increased. Other investigators have implicated epidermal growth factor (Wiesen et al. *1999*), transforming growth factor β (Daniel et al. *1996*), hepatocyte growth factor (Soriano et al. *1998*), insulin-like growth factor 1 (Kleinberg et al. *2000*), and vascular endothelial growth factor (Pepper et al. *2000*) as being stromal factors that positively influence ductal growth. Rodent stroma, however, has been reported to be devoid of PRL receptors that would be required to effect such an indirect stimulation (Meister et al. *1992*; Ouhtit et al. *1993a*, *1993b*).

The different effects of the two forms of PRL in the mammary gland are probably the result of different signaling. These two forms of PRL have been shown to initiate different signaling cascades in Nb2 cells (Coss et al. *1999*) and in HC11 cells (Wu et al. *2001*). In HC11 cells, S179D PRL upregulates the short form of the PRL receptor and signals primarily through MAP kinase for superior β-casein gene expression (Wu et al. *2001*). Some activation of STAT5 through the Jak2/STAT5 pathway, however, is essential.

In conclusion, we have clearly shown individual effects of the two forms of PRL on the mammary gland. U-PRL promotes growth whereas S179D PRL (pseudophosphorylated PRL) inhibits growth and promotes a differentiated function in this tissue. It is probably important that both forms are present throughout development of the mammary gland. Excesses of U-PRL might otherwise result in uncontrolled growth, whereas excesses of phosphorylated PRL would inhibit necessary growth and cell replacement. The proportion of U-PRL to phosphorylated PRL must change during development of the mammary gland in preparation for lactation, such that growth initially predominates and is later superceded by differentiated function. A substance such as S179D PRL, which inhibits growth and promotes a differentiated function, may have potential in the treatment or prevention of breast cancer.

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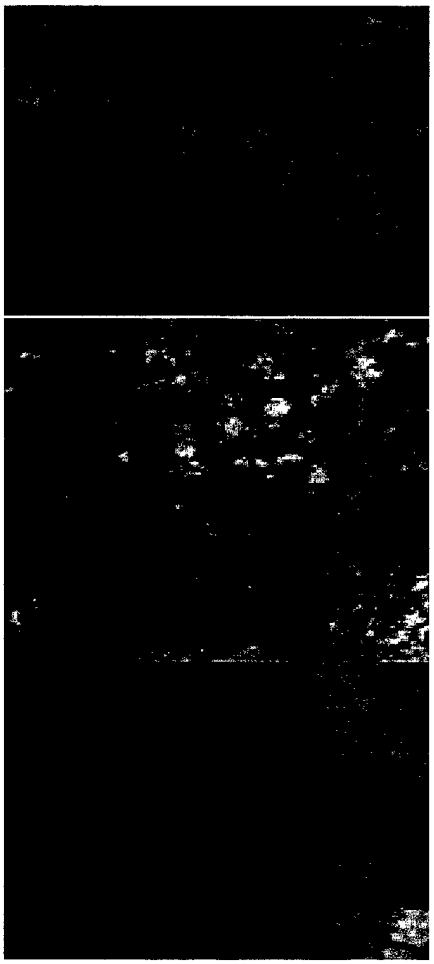


Figure 1

Figure 2

Figure 3

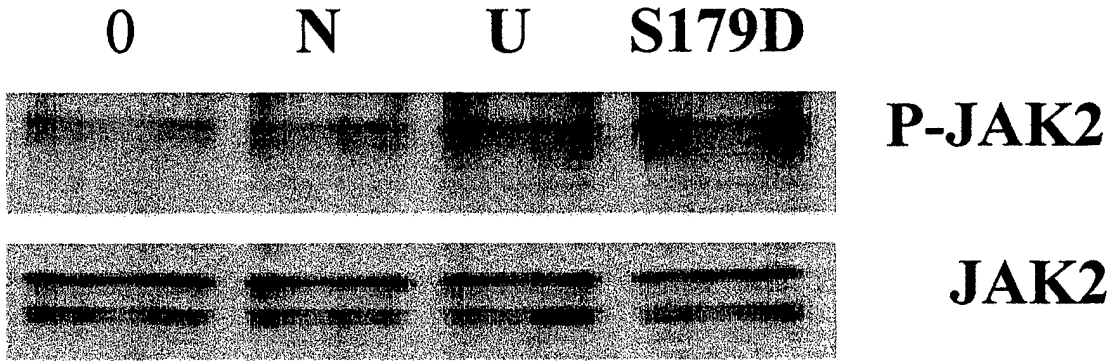
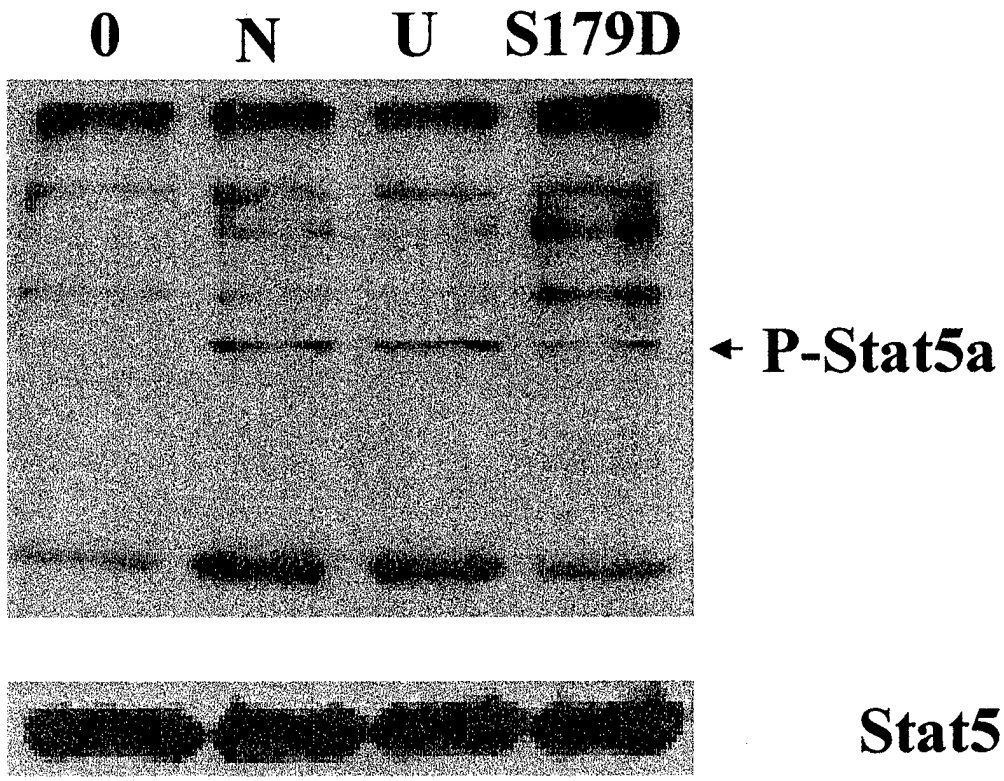


Figure 4



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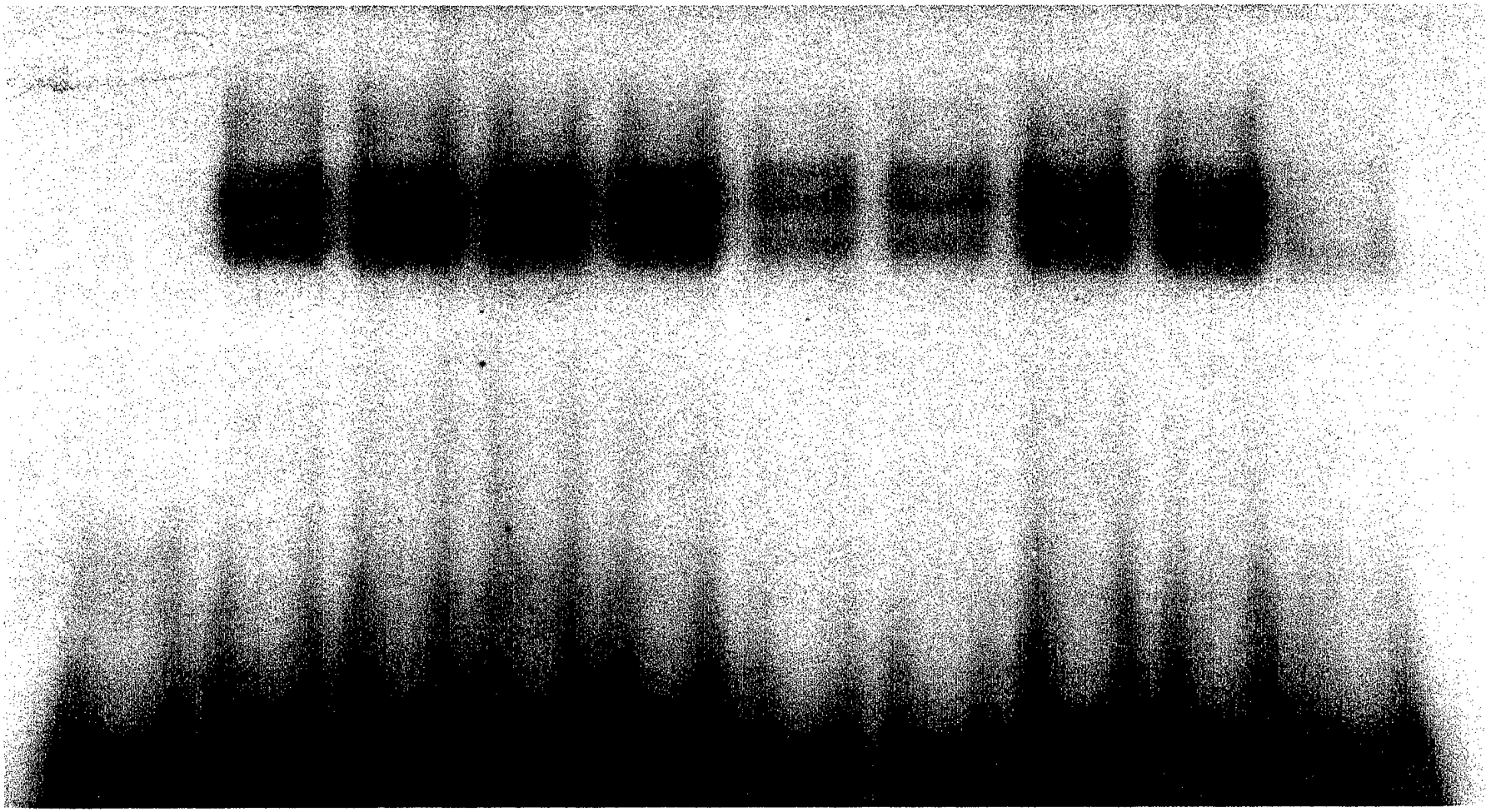


Oligo: TTCNNNGAA (β-casein GAS site)

Figure 6

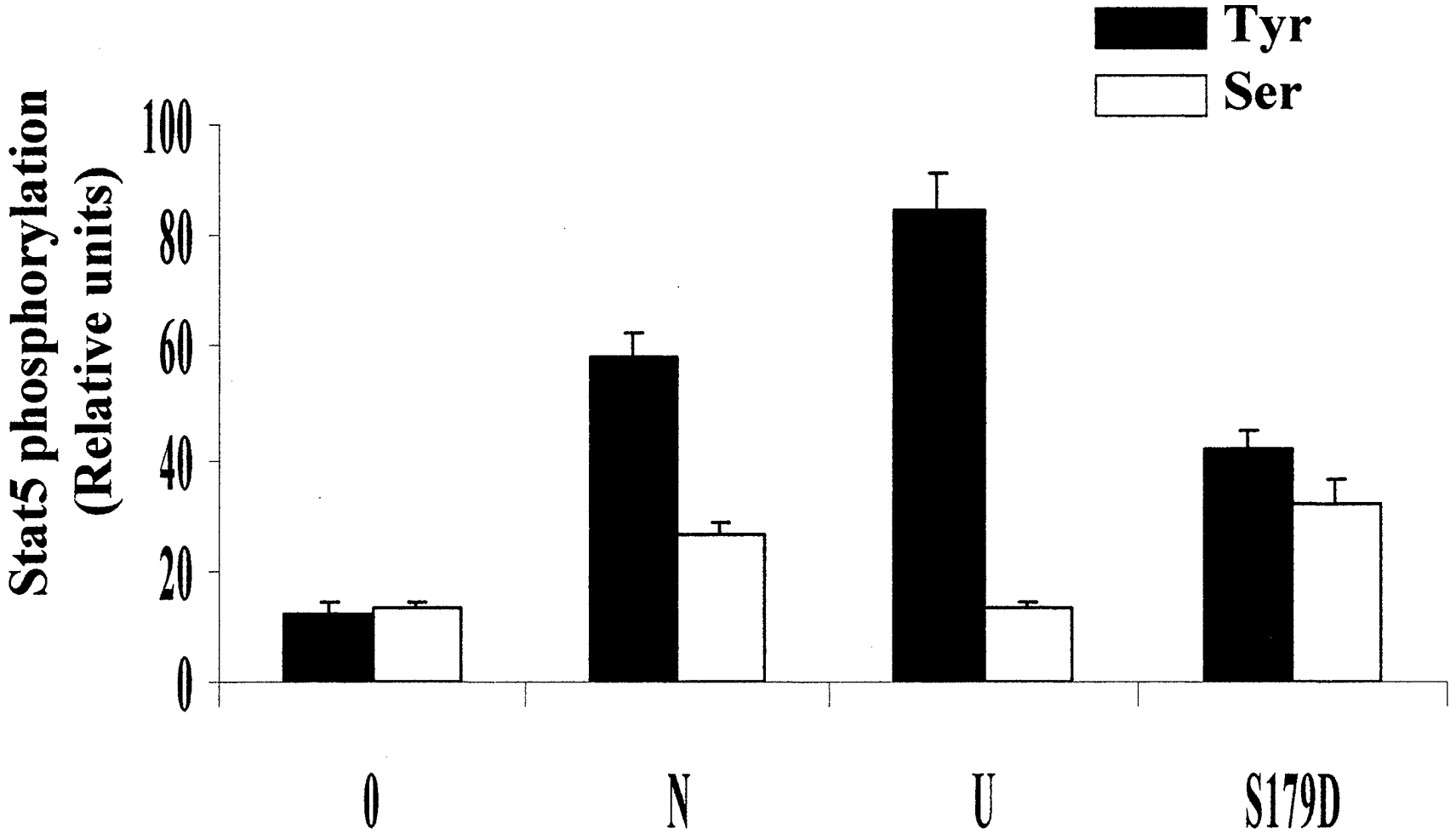

Tyr
Ser
Stat5 phosphorylation
(Relative units)
100
80
60
40
20
0
0
N
U
S179D

Figure 7

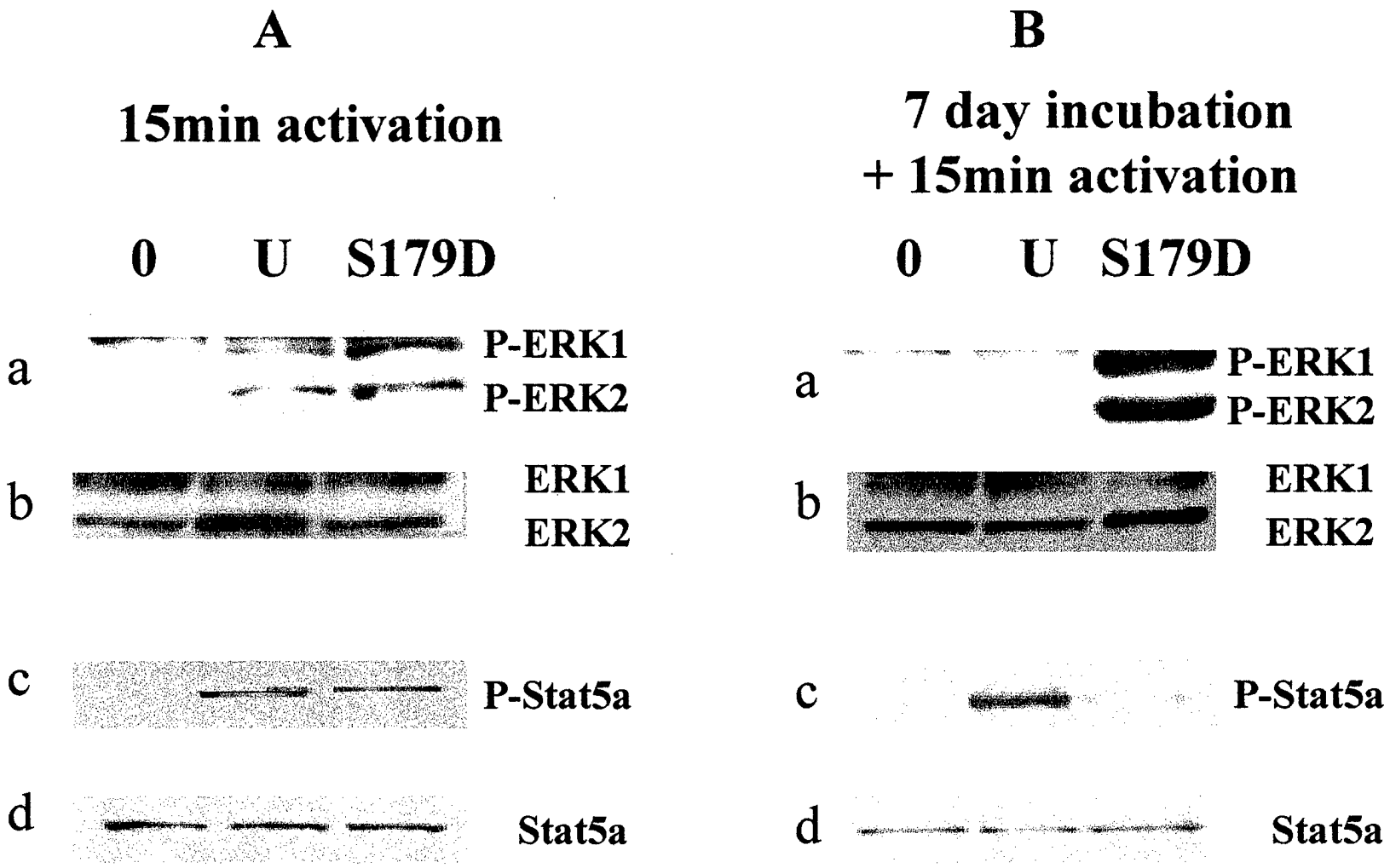

A
15min activation
0
U
S179D
a
P-ERK1
P-ERK2
b
ERK1
ERK2
c
P-Stat5a
d
Stat5a
B
7 day incubation
+ 15min activation
0
U
S179D
a
P-ERK1
P-ERK2
b
ERK1
ERK2
c
P-Stat5a
d
Stat5a

Figure 8

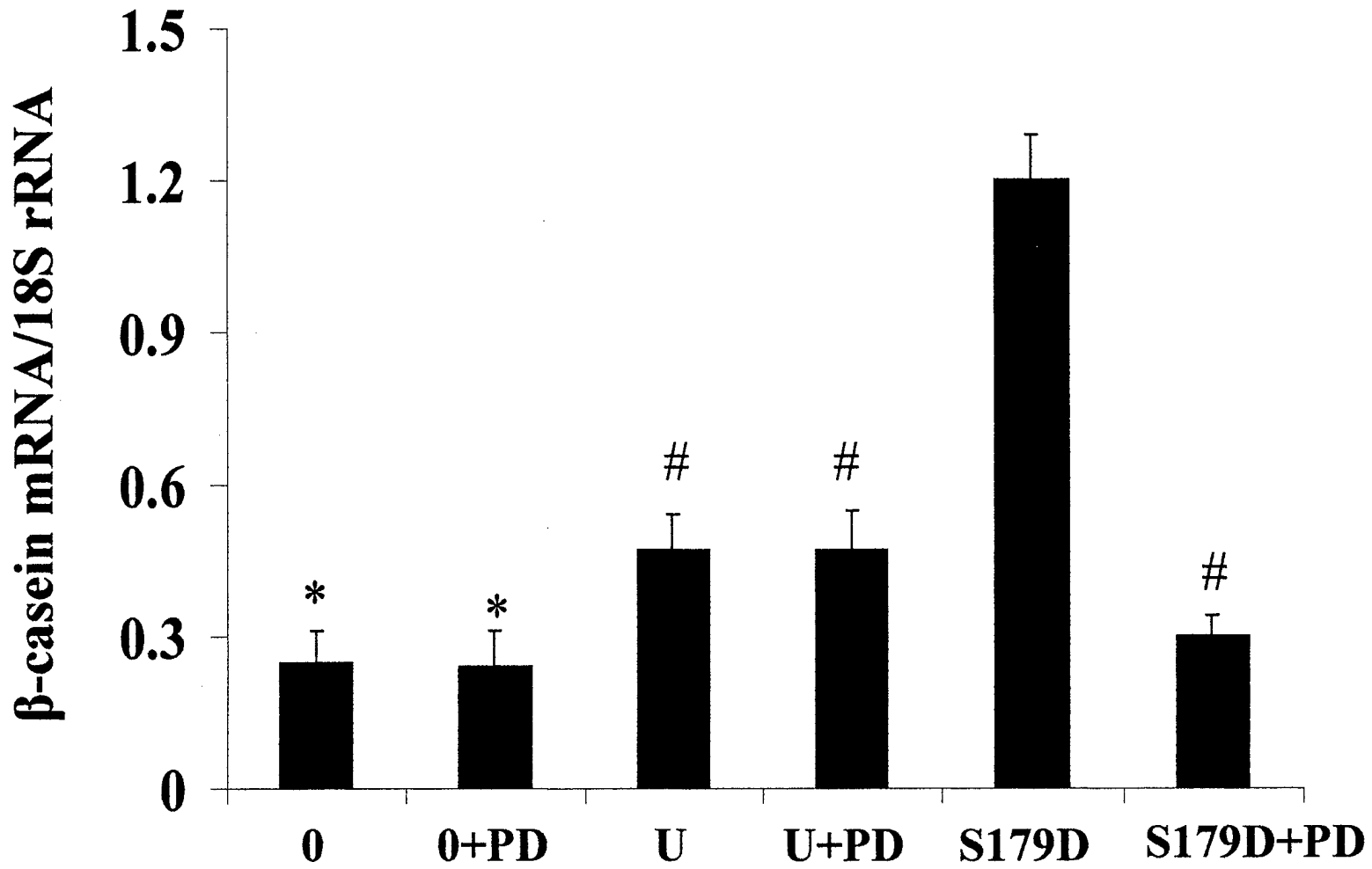
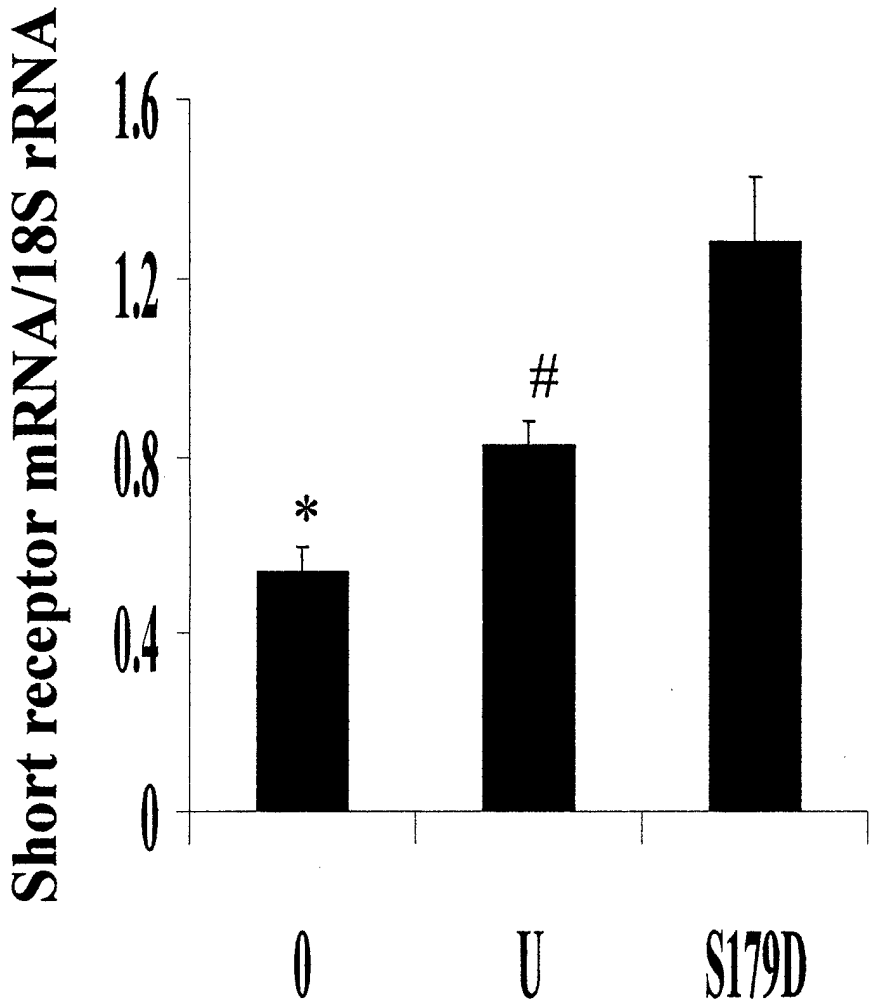


Figure 9


Short receptor mRNA/18S rRNA
1.6
1.2
0.8
0.4
0
*
#
0
U
S179D


0 U S179D
Short receptor
18S rRNA

Figure 10